

AD _____

AWARD NUMBER DAMD17-97-1-7086

TITLE: Estrogenicity of Medicinal Botanicals

PRINCIPAL INVESTIGATOR: Patricia K. Eagon, Ph.D.

CONTRACTING ORGANIZATION: Veterans Research Foundation of Pittsburgh
Pittsburgh, Pennsylvania 15240

REPORT DATE: June 1998

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

*Form Approved
OMB No. 0704-0188*

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY <i>(Leave blank)</i>			2. REPORT DATE June 1998		3. REPORT TYPE AND DATES COVERED Annual (1 Jun 97 - 31 May 98)	
4. TITLE AND SUBTITLE Estrogenicity of Medicinal Botanicals			5. FUNDING NUMBERS DAMD17-97-1-7086			
6. AUTHOR(S) Eagon, Patricia K., Ph.D.						
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Veterans Research Foundation of Pittsburgh Pittsburgh, Pennsylvania 15240			8. PERFORMING ORGANIZATION REPORT NUMBER			
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research And Materiel Command ATTN: MCMR-RMI-S 504 Scott Street Fort Detrick, Maryland 21702-5012			10. SPONSORING / MONITORING AGENCY REPORT NUMBER			
11. SUPPLEMENTARY NOTES			19981210 090			
12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited			12b. DISTRIBUTION CODE			
13. ABSTRACT <i>(Maximum 200 words)</i> Medicinal botanicals [plant extracts] have been used for centuries to relieve various gynecological symptoms, and are of increasing interest to those seeking alternative health care and self-treatment. However, women who have or are at risk for breast cancer pose a particular problem when using such materials, since little is known about their safety, potency, and hormonal properties. We tested extracts for estrogenic potency using several different criteria. In an <i>in vitro</i> competitive estrogen receptor (ER) binding assay, significant estrogenicity was observed with dang gui, licorice, blue and black cohosh, vitex and hops extracts. In an <i>in vitro</i> assay for ER-mediated transactivation, licorice, hops, and dang gui induced reporter gene expression in a dose-dependent manner. Some extracts were tested further for estrogenicity <i>in vivo</i> in ovariectomized female rats. Vitex, dang gui, and cohosh resulted in uterine weight increases. Serum LH levels were also quantitated to evaluate hypothalamic/pituitary response; LH levels were decreased by hops, vitex and cohosh. These studies verify that certain medicinal botanicals demonstrate measurable and significant estrogenic activities in several assays. The estrogenicity of such extracts may be clinically useful; conversely, these herbs might be avoided in conditions in which estrogens are contraindicated.						
14. SUBJECT TERMS Breast Cancer, phytoestrogens, botanicals, estrogenicity			15. NUMBER OF PAGES 15			
			16. PRICE CODE			
17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited			

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

PKE In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

Patricia K. Eagon

PI - Signature 6/29/98
Date

Table of contents:

Front cover:	1
Standard form #298	2
Foreword	3
Table of contents	4
Introduction	5
Body:	
Hypothesis	6
Methods	6
\b\Results	7
Discussion	13
Recommendations	14
Conclusions:	14
References:	15

INTRODUCTION:

Medicinal botanicals consisting of plant extracts have been used for centuries to relieve various gynecological symptoms, and are of increasing interest to those seeking alternative health care and self-treatment. However, women who have or are at risk for breast cancer pose a particular problem when using such materials, since little is known about their safety, potency, and hormonal properties. To determine whether these medicinal botanicals possess estrogenic activity, we focussed on several herbs that have been used to treat amenorrhea, dysmenorrhea (painful menstruation), and menopausal symptoms, especially the vasomotor symptom of hot flashes. Modern allopathic medicine treats hot flashes with exogenous estrogens, most typically derived from equine urine. Exogenous estrogen will reliably relieve hot flashes. Some herbs that relieve hot flashes have been shown to exert *in vivo* estrogenic effects. Others may affect feedback to the hypothalamic-pituitary-gonadal axis rather than direct binding to estrogen receptors in the end organ.

Two traditional Chinese remedies examined are dang gui (*Angelica sinensis*) and ginseng (*Panax ginseng*). Dang gui has a long history of use in gynecological maladies and during pregnancy, both as a female tonic and to promote easy delivery. Dang gui contains various coumarins and flavonoids, as well as many other compounds (1). Shiu chu ginseng also has a long history as an unparalleled revitalizing tonic. Among its many indications for use are pregnancy and puerperium, amenorrhea, metrorrhagia, and decreased sexual drive. Active compounds in ginseng include a range of steroid ginsenosides, among many others. Ginseng has been reported as estrogenic, but the mechanism remains unclear (2).

Hops (*Humulus lupus*), while used traditionally as a sedative, has a long history of notable estrogenic effects. In medieval Europe, workers in hops fields were observed to have increased sexual drive if female and decreased drive if male. The active phytoestrogens in hops have been reported as useful in relief of menopausal symptoms (3). Vitex, or chaste berry (*Vitex angus-castus*) is a Mediterranean plant traditionally used both to relieve menopausal symptoms and to decrease sexual drive. These contradictory indications have been explained by vitex's putative action at the pituitary rather than the estrogen receptor (4). Black cohosh (*Cimicifuga racemosa*) is a traditional American Indian cure for menstrual pain and menopausal discomfort. Cohosh extract is now popular in parts of Europe for use instead of estrogen replacement (5). Licorice root (*Glycyrrhiza glabra*) has also been used to menstrual and menopausal symptoms, and has been shown to contain high levels of glycyrrhizin, reputed to have both estrogenic and antiestrogenic properties and to interfere with metabolism of sex steroids, as well as the common coumarins and isoflavonoids (6-8).

Our work to date involved testing medicinal botanicals including those noted above using several different assessments *in vitro* and *in vivo* to determine their estrogenicity. These assays included an *in vitro* competitive estrogen receptor binding assay, and a reporter gene assay in rat uterine leiomyoma cells. *In vivo* effects were assessed by determining in ovariectomized (OVX) female rats uterine growth, serum ceruloplasmin levels and liver ceruloplasmin mRNA levels as measures of estrogenic response in liver, and serum LH levels as a measure of hypothalamic/pituitary response.

HYPOTHESIS:

Medicinal botanicals may have hormone agonist or antagonist activity, and therefore may stimulate or inhibit growth and hormone-mediated cellular events.

Understanding the mechanisms of action of these agents is critical in determining their efficacy in treatment of gynecological maladies, their safety of use, particularly in women with or at high risk for breast cancer.

METHODS:

Extract preparation: dried or fresh herbs were cold-extracted using ethanol and distilled water using a ratio (w/w) of 1:1.5 to 1:4 parts plant material. Extracts were stored in dark glass bottles. To the extent possible, the same batch of extract was used for all experiments shown. Plant extracts tested in all assays were dang gui (*Angelica sinensis*); black cohosh (*Cimicifuga racemosa*); hops (*Humulus lupulus*); vitex berry (*Vitex agnus-castus*); and Chinese ginseng (*Panax ginseng*). Extracts tested in some assays to date were: American ginseng (*Panax quinquefolium*); blue cohosh (*Caulophyllum thalictroides*); licorice root (*Glycyrrhiza uralensis*); raspberry leaf (*Rubus idaeus*); squaw vine (*Mitchella repens*); and wild yam root (*Dioscorea villosa*).

Estrogen receptor (ER) assay: the competitive ER binding assay using radiolabeled estradiol (E₂) has been published (9-12). Briefly, aliquots of cytosol prepared from ovariectomized female rat liver were incubated with 5nM [³H]-E₂ in the absence (control) and presence of test substances. The estrogens estradiol (E₂) and DES, androgen DHT, and phytoestrogen genistein were used in concentrations of 5nM-50μM, representing a range of 1X-10,000X the labelled E₂. The individual plant extracts were tested from dilutions of 1/20th to full-strength. The mixtures were incubated at 4°C overnight, and bound ligand was separated from free by treatment with dextran-coated charcoal (9).

Reporter gene transactivation: Transformed estrogen-responsive cells derived from an Eker rat uterine leiomyoma were transiently cotransfected with the firefly luciferase reporter gene linked to a estrogen-responsive promoter element (vitellogenin ERE), along with human estrogen receptor and a constitutive β-galactosidase reporter. Triplicate wells of transfected cells were washed and incubated for 40 hours in serum-free, phenol red-free medium in the presence or absence of test compound, then harvested and assayed for reporter gene activity normalized to activity of the constitutive reporter. Fold induction in treated samples is calculated as normalized reporter gene activity (mean of three wells) divided by activity in basal medium (untreated). A representative experiment is shown. An average fold-induction of 9.2 can be achieved using 100pM 17-β-estradiol. These experiments were conducted in the laboratory of Dr. Deborah Swafford, of the MD Anderson Cancer Research Center, Smithville, TX.

Administration to rats: extracts were mixed in 75ml of a complete liquid diet (Results! Diets, BioServ Corp); each ovariectomized female rat received the equivalent of a 1/3 human dose (500 µL) daily for 18-30 days. Each treatment group had 2-5 rats; control rats consisted of 2 intact and 3 OVX females. Rats were sacrificed, body & uterus weights measured, and serum collected. RNA was extracted from liver using standard methods (13). Serum ceruloplasmin measurements were done using the *p*-phenylamine-diamine oxidation method (14). Serum LH levels were determined by the Reproductive Medicine Core Facility at the University of Pittsburgh (15).

RESULTS:

Competitive ER binding assay: Figure 1 demonstrates the effect of the potent estrogens estradiol (E_2) and diethylstilbestrol (DES), the phytoestrogen genestein, and the androgen dihydrotestosterone (DHT) on the binding of radiolabeled E_2 to the ER. As noted by the decrease of E_2 binding with increasing hormone concentration, E_2 , DES, and genestein all appear to bind to the ER. The androgen DHT does not compete and therefore is not expected to bind to the ER.

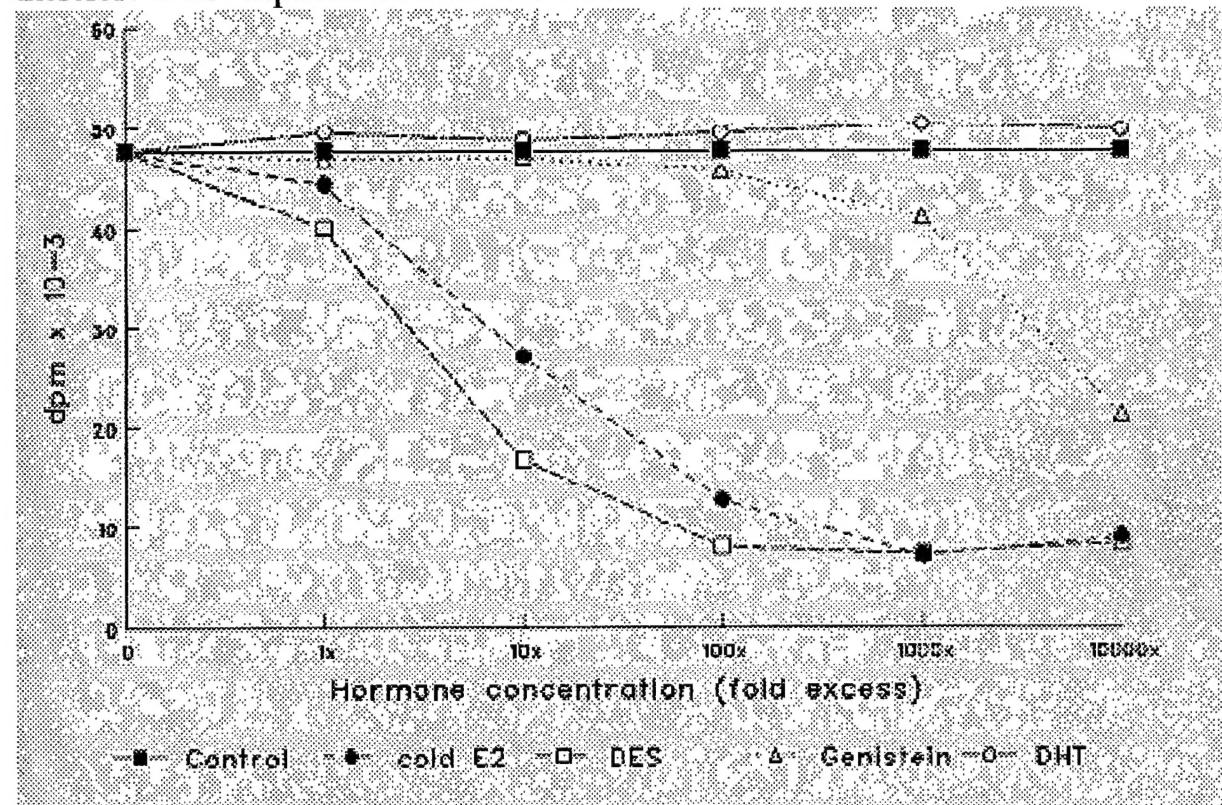


Figure 1: Competition of various sex hormones in an *in vitro* rat liver cytosolic estrogen receptor (ER) binding assay. Cytosol containing known levels of ER were incubated with [3 H]- E_2 in the absence and presence of increasing concentrations of potential competing hormones, including nonradioactive estradiol (E_2), diethylstilbestrol (DES), genestein, and dihydrotestosterone (DHT). A concentration-dependent decrease in [3 H]- E_2 binding in the presence of competitor indicates an interaction with the ER.

As shown in Figure 2, two extracts, hops and dang gui, competed about to the extent of genestein (Figure 1), and vitex competed slightly. A variation of this assay was used to test other extracts; several others, namely licorice, hops, blue cohosh, and vitex, also showed strong competition for ER binding (Figure 3).

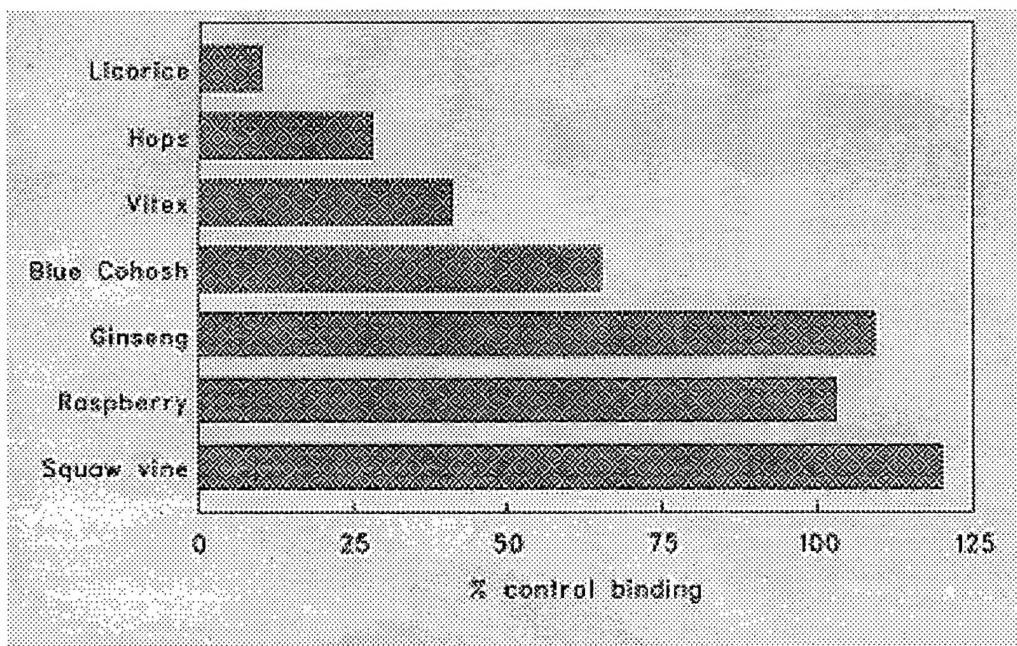
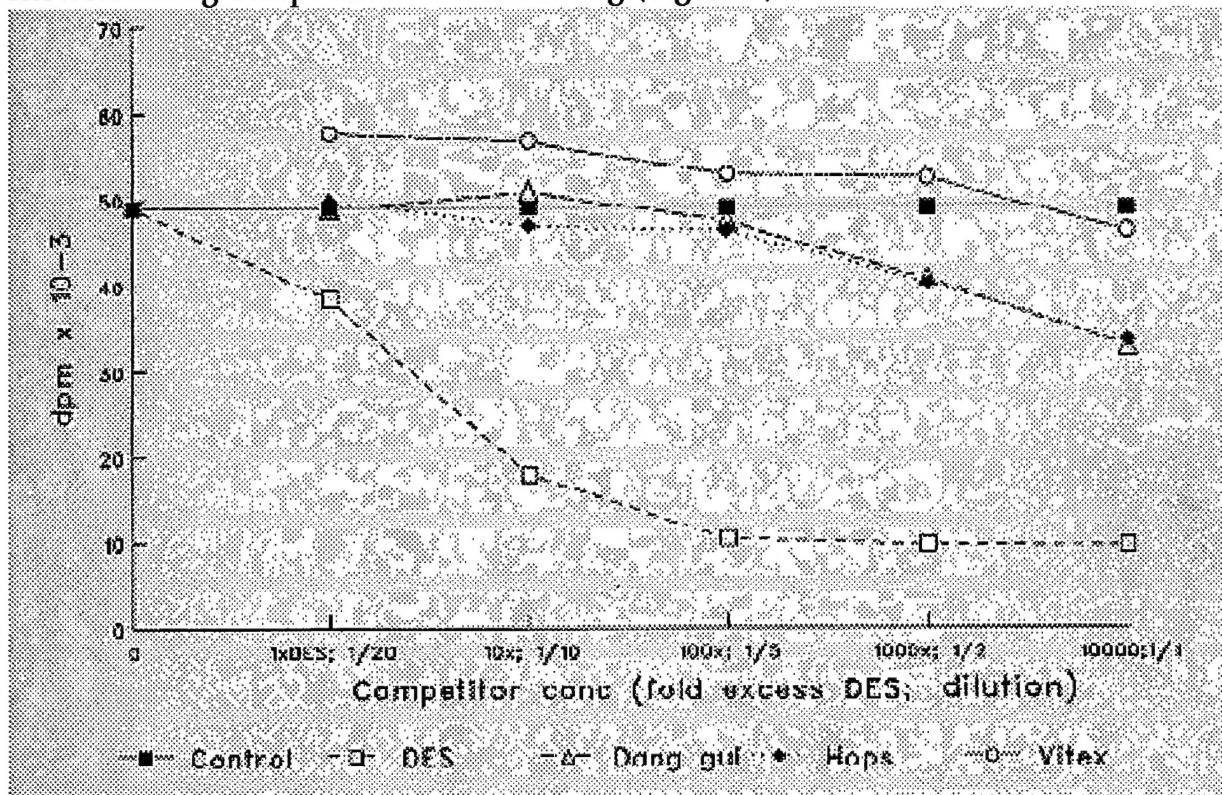


Figure 2 (top previous page): Competition of various plant extracts in an *in vitro* rat liver cytosolic ER binding assay. Cytosol containing known levels of ER were incubated with [³H]-E2 in the absence and presence of increasing concentrations of plant extracts, including vitex, dang gui, and hops. Diethylstilbestrol (DES) was included as a positive control. A concentration-dependent decrease in [³H]-E2 binding in the presence of competitor indicates an interaction with the ER.

Figure 3 (bottom previous page): Competition of various plant extracts in an *in vitro* rat liver cytosolic ER binding assay. In an assay slightly modified from that shown in Figures 1 and 2, other plant extracts were tested. The bars represent the maximum competition expressed by each extract.

Reporter gene transactivation: Transformed estrogen-responsive cells derived from an Eker rat uterine leiomyoma were transiently cotransfected with the firefly luciferase reporter gene linked to a estrogen-responsive promoter element (vitellogenin ERE), along with human estrogen receptor and a constitutive β -galactosidase reporter. Triplicate wells of transfected cells were washed and incubated for 40 hours in serum-free, phenol red-free medium in the presence or absence of test compound, then harvested and assayed for reporter gene activity normalized to activity of the constitutive reporter. As shown in Figure 4, hops, licorice, and dang gui demonstrated estrogen-responsive increases in gene expression as compared to basal medium with no additions.

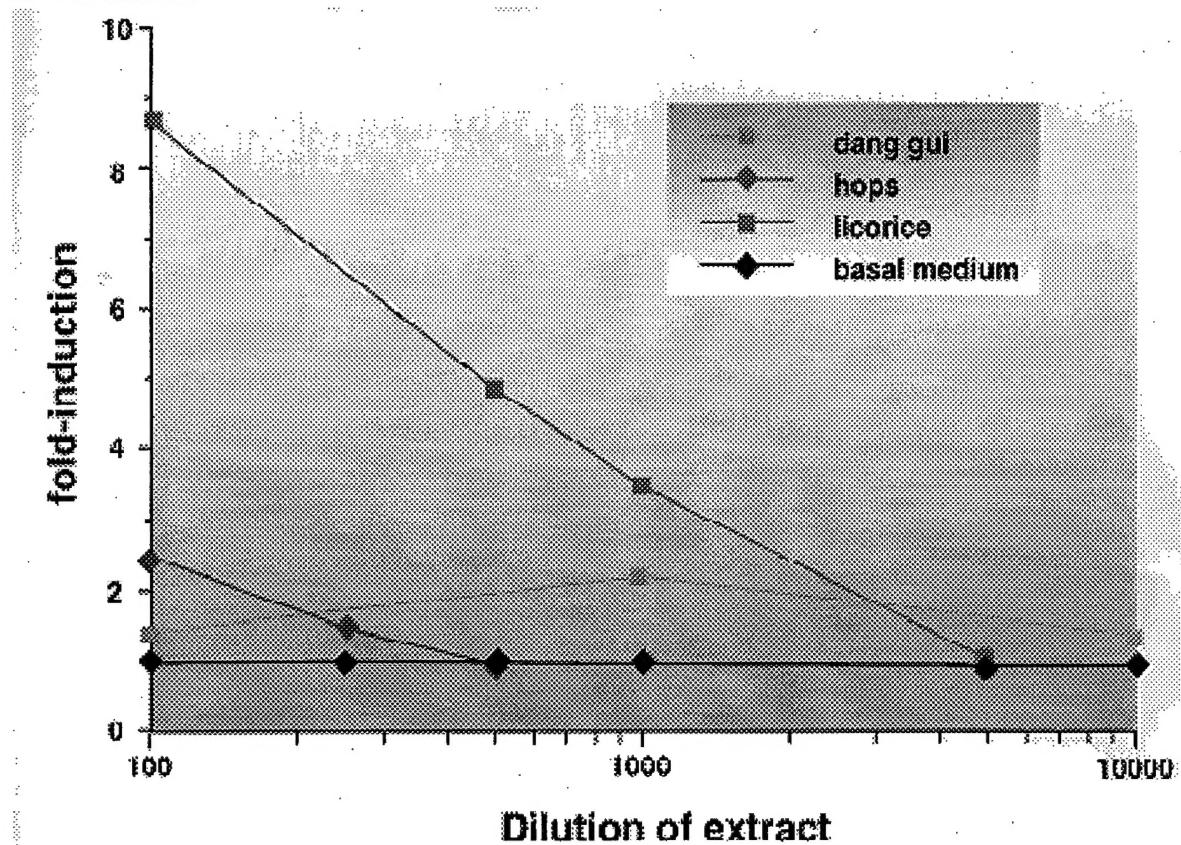


Figure 4: Reporter gene transactivation by plant extracts. Transformed estrogen-responsive cells derived from an Eker rat uterine leiomyoma were incubated with various plant extracts using several dilutions as shown. An estrogenic response was considered to be positive when the fold induction of the estrogen-responsive gene was greater than that with no additions.

Uterine weight increases: As shown in Figure 5, administration of extracts to OVX female rats for 30 days resulted in increases in uterine weight; significant increases, as compared to untreated rats, were demonstrated with black cohosh, dang gui, and vitex. Ginseng increased uterine weight slightly but not significantly.

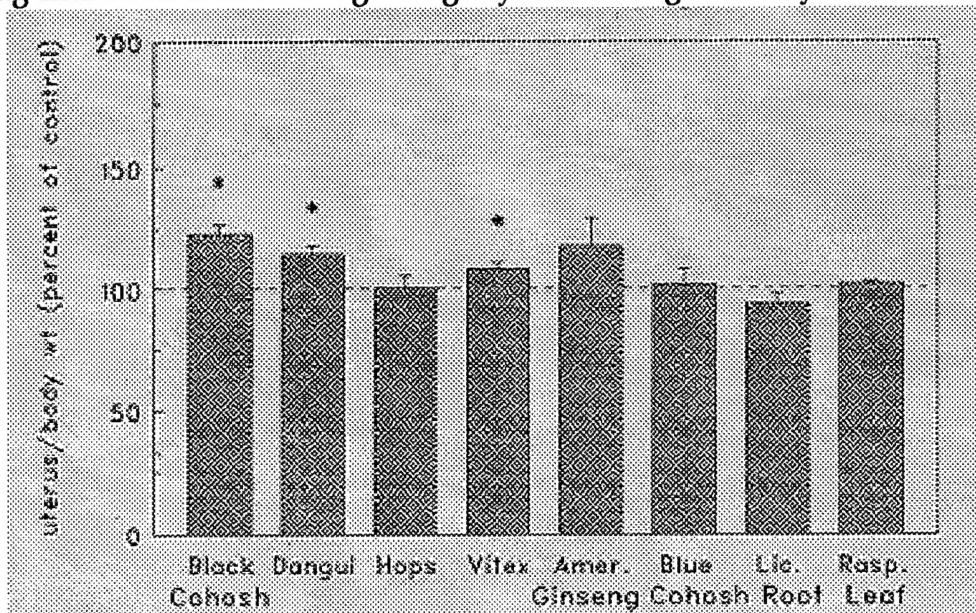


Figure 5: Effect of plant extracts administered in diet on uterine weight. Ovariectomized female rats (5 per group) were fed diets containing the plant extracts shown or no extract for 30 days. At sacrifice, uteri were dissected and weighed. The results from animals fed extracts are compared to those from rats which received no extracts in the diets (horizontal dotted line, 100%). The asterisks indicate $p < 0.05$.

Induction of ceruloplasmin expression: The liver responds to estrogens by an increase in synthesis and secretion of serum ceruloplasmin. As seen in Figure 6, serum ceruloplasmin increases after a 30 day treatment with hops and dang gui, as compared

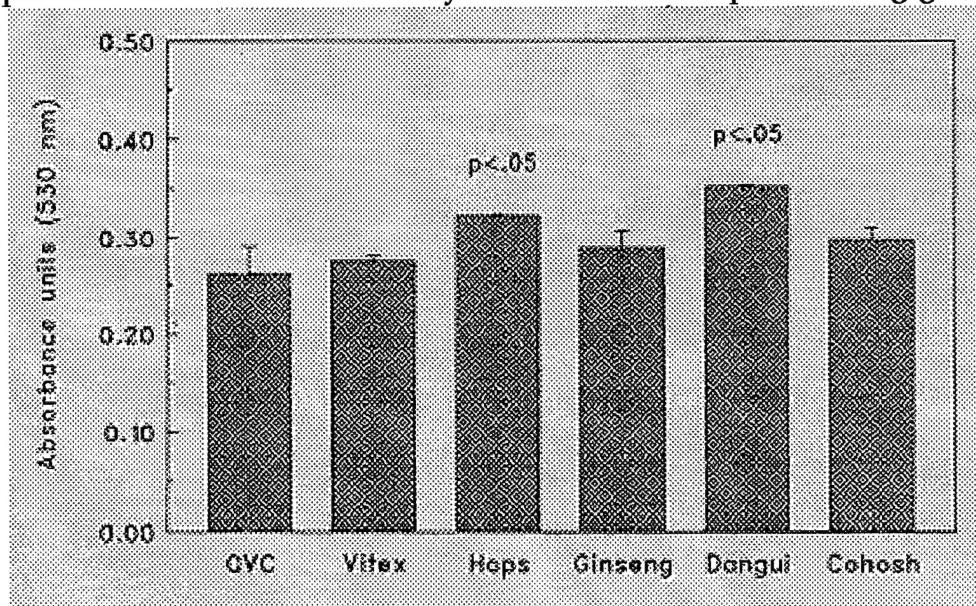


Figure 6: legend on following page.

Figure 6 (previous page): Effect of plant extracts administered in diet on serum ceruloplasmin.
 Animals were treated as noted in Figure 5 legend; an increase in serum ceruloplasmin represents an estrogenic response by liver of these animals.

to that in the untreated OVC (OVC) group, but other extracts have no effect in this time period. The magnitude of increase with these two extracts is similar to that seen after a 15 day treatment with estradiol or tamoxifen, which is estrogenic in liver (Figure 7).

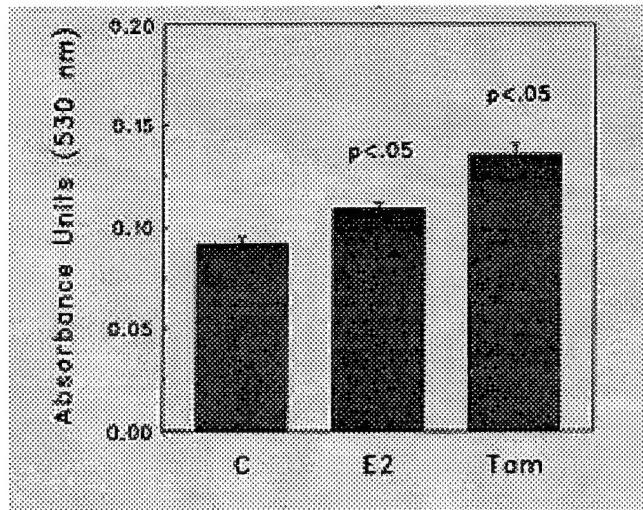


Figure 7: Effect of administration of estrogens on serum ceruloplasmin. Ovariectomized female rats (5 per group) were treated by implants containing estradiol (E2) or tamoxifen (Tam) for 15 days; an increase in serum ceruloplasmin represents an estrogenic response by liver.

Ceruloplasmin mRNA is induced in the liver significantly by vitex, hops, dang gui, and ginseng, and slightly by black cohosh (Figure 8). It is not clear why a stronger effect is seen at the mRNA level than at the protein level; perhaps turnover of either mRNA or protein is affected by these agents.

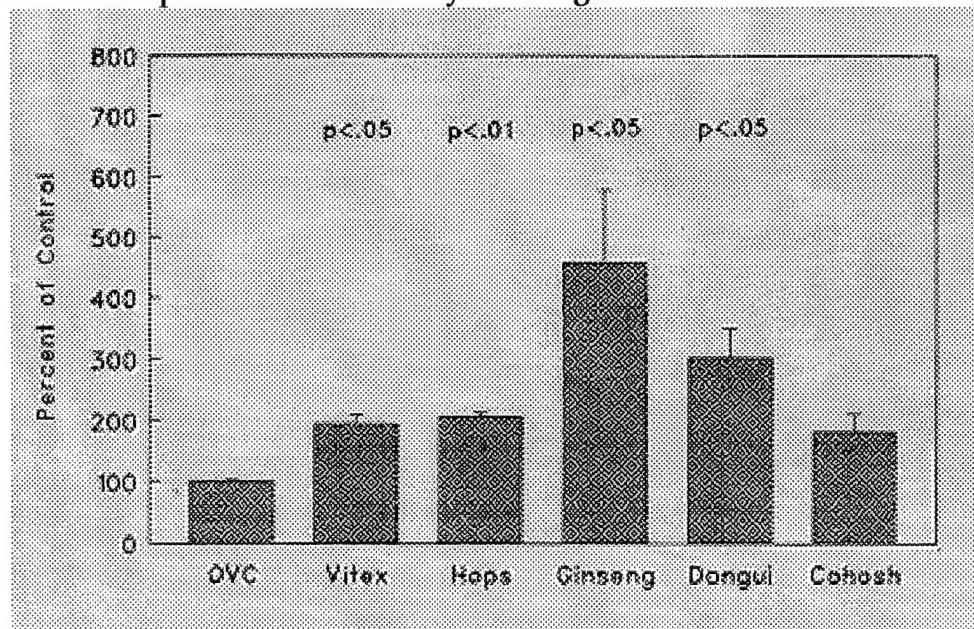


Fig 8: legend next page.

Figure 8 (previous page): Effect of plant extracts administered in diet on liver ceruloplasmin mRNA. Animals were treated as noted in Figure 5 legend; an increase in ceruloplasmin mRNA represents an estrogenic response by liver of these animals.

Effect of extracts on serum LH levels: Serum LH levels are high in OVX female rats as compared to intact females; thus, a measure of estrogenicity at the level of the hypothalamus/pituitary is a reduction in LH levels. As demonstrated in Figure 9, a 30-day exposure to several extracts results in a reduction in LH, namely vitex, hops, dang gui, and black cohosh.

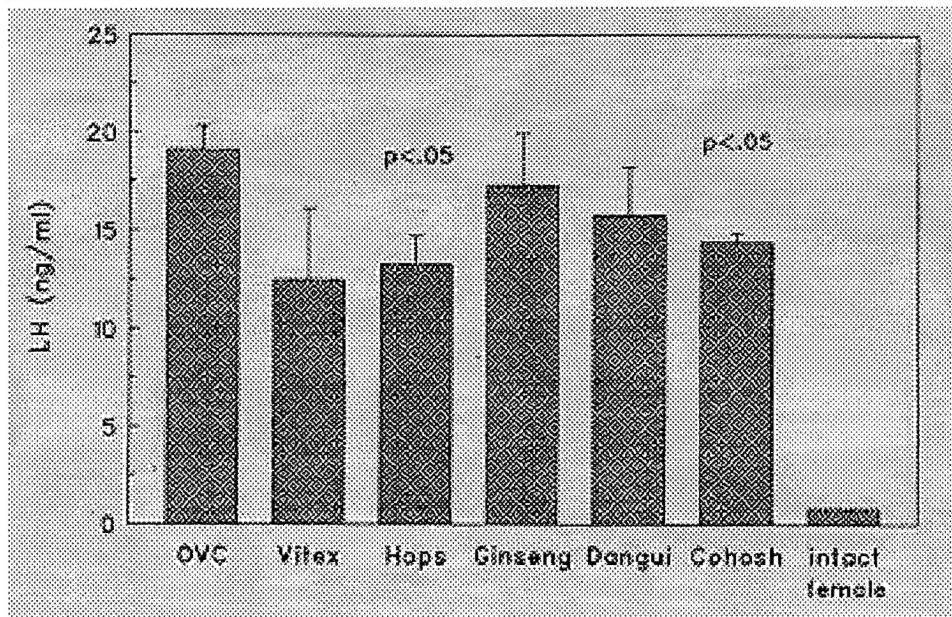


Figure 9: Effect of plant extracts administered in diet on serum LH levels. Animals were treated as noted in Figure 5 legend; a decrease in serum LH represents a hypothalamic/pituitary estrogenic response in these animals. Shown for comparison are values from untreated ovariectomized control rats (OVC) and rats which were not ovariectomized (intact).

These results, taken together, demonstrate that many of these medicinal botanicals are indeed estrogenic. Table I presents a comparison of all testing to date. It is clear that not all extracts are estrogenic in all assays.

TABLE I: A comparison of estrogenicity of extracts in several assays.

Extract	ER Assay	Uterus Wt	Serum CP	Reporter geneassay	Serum LH
Vitex	±	+	±	-	+
Hops	+	-	+	+	+
Ginseng (Chi)	-	-	±	-	-
Ginseng (Am)	-	±	nd	-	-
Dang gui	+	+	+	±	±
Black cohosh	-	+	±	-	+
Blue cohosh	+	-	nd	-	nd
Licorice	+	-	nd	+	nd
Raspberry	-	-	nd	-	nd
Squaw vine	-	nd	nd	-	nd
Wild yam root	-	nd	nd	-	nd

KEY: (+) strong; (±) weak; (-) not estrogenic; (nd) not done.

DISCUSSION:

A number of different types of assays were used to determine whether the extracts tested demonstrated estrogenicity. Since a number of factors influence all assays, it was our opinion that several methods of testing was warranted to best evaluate these products. Of all the extracts tested, only one, dang gui, proved to be estrogenic by all criteria. However, several others demonstrated estrogenicity in most assays; these include vitex, hops, licorice and black cohosh.

Several points should be considered when evaluating this information. First, differences in results may be a function of the assay. Differences in estrogenicity as measured *in vitro* and *in vivo* might reflect several possibilities: metabolism *in vivo* to more or less potent agents; mechanisms not involving the ER; or alterations in activity of sex steroid metabolizing enzymes, which was not addressed in this work.

Metabolism of extract components is likely when administered *in vivo*, but less likely in the *in vitro* ER binding assay. The dietary method of administration most closely represents human consumption of these extracts. Further, the uterus weight and CP measurements represent a relatively *short* exposure to the extracts (18-30 days). These parameters may increase upon longer treatment; also, extracts which do not show an early effect might become positive with longer exposure.

RECOMMENDATIONS:

The work has proceeded without serious problems. Minor problems encountered involved quenching in quantitating radiolabel levels in the ER binding assay, due to the dark yellow/orange/brown color of the extracts. This problem was solved by modifying the assay so that the color is removed prior to scintillation counting. Another problem was that some of the extracts are bitter, and the rats would not eat the complete meal; this problem was solved by adding aspartame to the diet, since rats are fond of sweet food. We will continue as outlined in the Statement of Work without altering this schedule.

CONCLUSIONS :

Many of the medicinal botanicals tested appear to be estrogenic, although varying in potency from very active to only weakly so. In addition, the botanicals which are estrogenic may not affect all organs similarly. One extract, dang gui, is particularly potent in all assays used, although vitex, licorice, hops and cohosh demonstrate significant estrogenicity as well. It should be noted that beer contains hops, and licorice is commonly sold as candy.

Thus, women who are at risk for, or who have breast cancer, and in particular, estrogen receptor-positive disease, should not take such preparations without medical supervision. Certainly further testing is needed to determine the safety and efficacy of these agents in this particular group of women. In women not at risk, these preparations may be useful in relieving menopausal symptoms, but dosage should be monitored carefully. Certain of the herbs tested *in vivo* demonstrate potency even when administered for a short time, and in some assays, the results were similar to those seen with administration of physiological levels of estradiol. Although these botanicals are natural products, the danger of these materials should not be dismissed; when taken in high doses there is potential for harm.

REFERENCES:

1. Zhu DPQ: Dong quai. Am J Chin Med 15:117-125, 1987
2. Punnonen R, Lukola A: Oestrogen-like effect of ginseng. Br Med J 281: 1110, 1980.
3. Murray, M.T. *The Healing Power of Herbs*, 2nd ed., 1995, Prima Publishing Co., Rocklin CA. This comprehensive text cites hundreds of journal references.
4. Commission E Monograph: Agni cast fructus (chaste tree fruits). Translation by the American Botanical Council, Fort Worth TX, 1992
5. Duker EM: Effects of extracts from *Cimicifuga racemosa* on gonadotropin release in menopausal women and ovariectomized rats. Planta Medica 57: 420-424, 1991.
6. Kumagai A: Effect of glycyrrhizin by human intestinal flora. Planta Medica 48: 38-42, 1983.
7. Kraus S: the anti-estrogenic action of beta-glycyrrhetic acid. Exp Med Surg 27: 411-420, 1969.
8. Sharaf A, Goma N: Phytoestrogens and their antagonism to progesterone and testosterone. J Endocrinol 31: 289-290, 1965.
9. Eagon PK, Fisher SE, Imhoff AF, Porter LE, Stewart RR, Van Thiel DH, Lester R. Estrogen-binding proteins of male rat liver: influences of hormonal changes. Arch Biochem Biophys 201: 486, 1980.
10. Porter, L.E., Elm MS, Van Thiel DH, Eagon PK: Characterization and quantitation of human hepatic estrogen receptor. Gastroenterology 84: 704-712, 1983
11. Porter, LE, Elm MS, Van Thiel DH, Eagon PK: Hepatic estrogen receptor in human liver disease. Gastroenterology 92: 735-745, 1987.
12. Eagon PK, Francavilla A, DiLeo A, Elm MS, Gennari L, Mazzaferro V, Collella G, Starzl TE. Quantitation of estrogen and androgen receptors in hepatocellular carcinoma and adjacent normal human liver. Dig Dis Sci 36: 1303, 1991.
13. Eagon, PK, Elm MS, Epley MJ, Shinozuka H, Rao KN: Sex steroid metabolism and receptor status in hepatic hyperplasia and cancer in rats. Gastroenterology 110: 1199-1207, 1996.
14. Sunderman FW, Nomoto S. Characterization of an improved method for the measurement of serum ceruloplasmin. Clin Chem 16:903-910, 1970.
15. Gavaler JS, Rosenblum ER, Van Thiel DH, Eagon PK, Pohl CR, Campbell IM, and Gavaler J: Biologically active phytoestrogens are present in bourbon. Alcoholism: Clin. Exp. Res. 11: 399-406, 1987.